In re the Application of:
Leon W.M.M. Terstappen, Galla Chandra Rao, Herman Rutner, and Paul A. Liberti
Application/Control Number: 09/801,471
Application Filed: March 7, 2001
Title: Labeled Cells For Use As An Internal Functional Control In Rare Cell Detection Assays.
Art Unit: 1642
Examiner: Karen A. Canella, Ph.D.

DECLARATION under Rule 132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 200231

Herman Rutner declares as follows:

1. I am a citizen of the USA, B.S. (Chemistry), M.S. (Organic Chemistry) from the Polytechnic University of Brooklyn, NY, residing at 50 South Penn Street, Hatboro, PA 19040, and co-inventor of the above patent application (Publication No.: US 2001/0018192 A1, Application No.: 09/801,471).
2. I was directly involved in the experimentation and optimization processes leading to the singly labeled control cells of the above application, specifically the pre-labeling of cell membranes of cultured cancer cells with lipophilic carbocyanine dyes as described in Example 1. I was also involved in the conception and implementation of the dual or redundant pre-labeling of internal control cells.
3. We unexpectedly found that even traces of phosphate efficiently precipitate the cationic carbocyanine dyes to form agglomerates, manifested as faint Tyndall turbidity, make the membrane staining less efficient. Hence removal of all media or buffers containing phosphate was found to be critical in our staining protocols. The conventional stock solutions of the dyes in ethanol when added to the diluents also produce colloidal dye agglomerates or turbidity on brief standing that similarly decrease staining efficiencies or uniformity. We found that DMSO stock solutions of the dyes, diluted 1:100 with 5% mannitol, showed excellent solubility and stability, as judged by absence of Tyndall turbidity, for more than 1 hr or a time period exceeding the cell staining time. Mixing of the dilute dyes with an equal volume of washed cell suspension in diluent and incubation for about 30 min gave the highest staining efficiencies that we attributed to the absence of phosphate and the presence of 0.5% DMSO, a diffusion promoter, in the incubation mixture. This staining protocol is disclosed in Example 1 of the above application. Despite the excellent data showing 0 unstained control cells in 2000-3000 spiked control cells by flow cytometry (Fig 5-7 of the above application), we noted an unacceptably high level of dimly stained control cells in our microscope based CellSpotter analysis platform, particularly with control cells. The presence of dimly stained control cells is tolerable in flow analysis where classification of cells is on a log scale and a 10 fold difference (1 log unit) in staining intensity is tolerable. But such variations would cause potential

misclassification errors or false positive results that are unacceptable in microscopic imaging of rare cells. Hence the need for more efficient and uniform pre-labeling of control cells led to the conception of a dual pre-labeling approach, designated as "redundant labeling", which is an element of the present application. We developed this in response to a unique need by what would appear foolhardy by spiking a pre-labeled target cells at levels up to 10-100x higher than the number of identical target cells in a specimen, i.e. containing as few as 1-2 target cells, with the expectation of being able to fully discriminate them. Conventional pre-labeling with a single dye reduces the probability of unlabeled or dimly labeled control cells from about <1 cell per 1000 control cells (see Fig. 5 – 7 in present application). In contrast, redundant pre-labeling with two different dyes results in essentially zero or <1 unlabeled cells in 1 million control cells, thereby providing a practical solution to achieving the extremely high specificity required for discriminating spiked labeled target cells from endogenous target cells in rare cell detection by microscopic imaging. As shown in the attached copies of laboratory data (Figures A and B), mislabeling of 1 in every 100 spiked control cells (of 5000 added control cells) will result in a 100% probability of detecting the control cell as a false positive (target tumor cell). When the mislabeling is reduced to 1 in 1,000,000 (redundant labeling), the probability is reduced to 0.499% which is a satisfactory value for the Cell Spotter® Test. Figure C shows related e-mail discussing the practical application of relevant stains and the completeness of the staining process.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed: Herman Rutner Date: 8/14/03

Herman Rutner, MS
Principal Scientist
Witness: Ellen B. Turk

Notarial Seal
Ellen B. Turk, Notary Public
Bryn Athyn Boro, Montgomery County
My Commission Expires Aug. 16, 2003